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Title:

Pre-Exercise Breakfast Ingestion *versus* Extended Overnight Fasting Increases
Postprandial Glucose Flux after Exercise in Healthy Men

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RLT and J-PW conducted the research, RME, JTG, AH, HS, FK, and GAW
analyzed the data, RME and JTG performed the statistical analysis, RME and
JTG primarily wrote the paper, and all authors contributed to earlier versions of
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23 **Running Head:** Pre-exercise feeding and postprandial glucose flux

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27 **Trial Registration:** <https://clinicaltrials.gov/> [Number: NCT02258399]

ABSTRACT

Aims: To characterize postprandial glucose flux after exercise in the fed *versus* overnight fasted-state and to investigate potential underlying mechanisms.

Methods: In a randomized order, twelve men underwent breakfast-rest (BR; 3 h semi-recumbent), breakfast-exercise (BE; 2 h semi-recumbent before 60-min of cycling (50% peak power output) and overnight fasted-exercise (FE; as per BE omitting breakfast) trials. An oral glucose tolerance test (OGTT) was completed post-exercise (post-rest on BR). Dual stable isotope tracers ([U-¹³C] glucose ingestion and [6,6-²H₂] glucose infusion) and muscle biopsies were combined to assess postprandial plasma glucose kinetics and intramuscular signaling, respectively. Plasma intestinal fatty acid binding (I-FABP) concentrations were determined as a marker of intestinal damage. **Results:** Breakfast before exercise increased post-exercise plasma glucose disposal rates during the OGTT, from 44 g·120 min⁻¹ in FE [35 to 53 g·120 min⁻¹] (mean [normalized 95% CI]) to 73 g·120 min⁻¹ in BE [55 to 90 g·120 min⁻¹; $p = 0.01$]. This higher plasma glucose disposal rate was, however, offset by increased plasma glucose appearance rates (principally OGTT-derived), resulting in a glycemic response that did not differ between BE and FE ($p = 0.11$). Plasma I-FABP concentrations during exercise were 264 pg·mL⁻¹ [196 to 332 pg·mL⁻¹] lower in BE *versus* FE ($p = 0.01$). **Conclusion:** Breakfast before exercise increases post-exercise postprandial plasma glucose disposal, which is offset (primarily) by increased appearance rates of orally-ingested glucose. Therefore, metabolic responses to fed-state exercise cannot be readily inferred from studies conducted in a fasted state.

52 **Key words:** Breakfast; Exercise; Insulin sensitivity; Glycemia; Metabolism

53 INTRODUCTION

54 Postprandial glycemia is a strong predictor of future mortality and morbidity.
55 Even in people without diabetes, those with greater blood glucose excursions
56 after feeding are at an increased risk of cardiovascular disease (47, 48). This
57 glycemic response to food ingestion is dictated by blood glucose kinetics (i.e.
58 the balance between the rates of glucose appearance into blood and glucose
59 disposal from blood into peripheral tissues). Exercise potently increases
60 glucose disposal from the blood into skeletal muscle (52), and regular exercise
61 is therefore recommended as a lifestyle strategy to improve glycemic control.

62

63 Habitual responses to exercise and nutrition are however, the culmination of not
64 only chronic adaptations, but also the acute effects of each exposure to these
65 daily behaviors (5, 6, 22). For example, each bout of exercise potently
66 stimulates post-exercise insulin sensitivity and muscle glucose uptake (52).
67 However, despite increases in blood glucose disposal rates, endurance-type
68 exercise does not always reduce postprandial glucose excursions in the post-
69 exercise period (20, 54). The finding that postprandial blood glucose
70 concentrations are not lowered post-exercise is because when exercise is
71 performed (at least in the fasted state), the increase in postprandial blood
72 glucose disposal after exercise can be offset - *and even superseded* - by
73 increases in both endogenous and meal-derived blood glucose appearance
74 rates (34, 54).

75

76 Whilst fasting prior to laboratory trials is common in order to control for baseline
77 metabolic status, these conditions may preclude the application of findings to
78 situations most representative of daily living. For example, most people living in
79 developed countries spend the majority of a typical day in the postprandial state
80 (13, 55). Therefore, most eating occasions and exercise sessions will take place
81 in the context of this postprandial situation (23). It has previously been shown
82 by others that plasma glucose fluxes *during* exercise (16), and by us that
83 plasma glucose *concentrations* after exercise (24), are elevated by pre-exercise
84 feeding. However, the effect of prior feeding on *post-exercise* plasma glucose
85 flux has never been assessed. Therefore, there is a distinct lack of
86 understanding regarding postprandial glucose kinetics under scenarios that are
87 most representative of daily living, and it may not be valid to generalize existing
88 observations of exercise in the fasted state. Moreover, an understanding of the
89 underlying mechanisms responsible for any differences in postprandial glucose
90 flux post-exercise, with prior feeding *versus* fasting, is still required.

91
92 This study therefore aimed to characterize postprandial plasma glucose kinetics
93 after: 1) breakfast and rest; 2) breakfast and exercise and 3) overnight fasted-
94 state exercise, while also exploring potential mechanisms (intramuscular
95 signaling and markers of intestinal damage) to explain any differences in
96 glucose flux between these conditions.

97 MATERIALS AND METHODS

98 Ethical Approval

99 All trials were undertaken at the University of Bath (Bath, UK) in accordance
100 with the Declaration of Helsinki. The study was approved by the National Health
101 Service South-West Research Ethics Committee (reference: 15/SW/0006) and
102 registered at clinicaltrials.gov as NCT02258399. Written, informed consent was
103 obtained from all participants prior to their participation.

104

105 Study Design

106 This study was a randomized cross-over design (randomization performed by
107 JTG with Research Randomizer version 3.0, <http://www.randomizer.org/>).
108 Preliminary testing was followed by three trials (separated by > 7 d), namely,
109 breakfast-rest (BR), breakfast-exercise (BE) and overnight fasted-exercise (FE).
110 A schematic for the study protocol is shown in **Figure 1**. For all trials
111 participants arrived at the laboratory after a 12 to 14-h overnight fast. In BR, a
112 porridge breakfast was consumed, followed by 3 h of rest, and then a 2-h oral
113 glucose tolerance test (OGTT). In BE, the same breakfast was consumed,
114 before 2 h rest and 60 min of cycling, prior to the OGTT. In FE, breakfast was
115 omitted but the trial otherwise replicated BE. By necessity of design (food
116 intake/exercise) the intervention was open label. Within-lab testing conditions
117 were not different across the trials ([mean \pm SD] ambient temperature [$23.7 \pm$
118 0.5 °C on BR, 23.7 ± 0.6 °C on BE, 23.6 ± 0.7 °C on FE] and barometric
119 pressure [734 ± 5 mmHg on BR, 736 ± 6 mmHg on BE, 736 ± 5 mmHg on FE];
120 all $p > 0.05$).

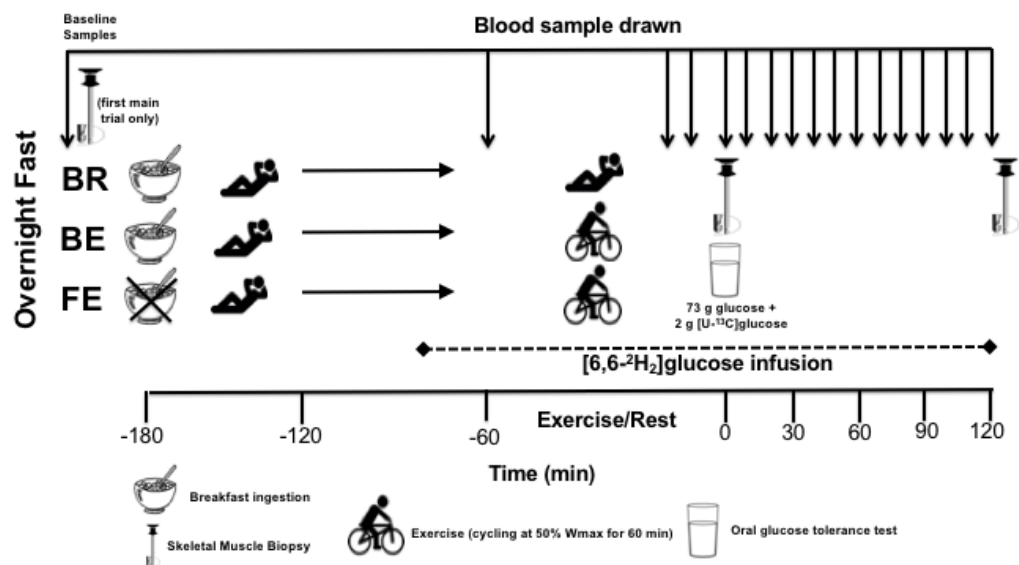


Figure 1. Protocol schematic. An oral glucose tolerance test was conducted after breakfast followed by rest (BR), breakfast followed by exercise (BE), or extended overnight fasting followed by exercise (FE). Dual stable isotope tracers ([U-¹³C] glucose ingestion and [6,6-²H₂] glucose infusion) and muscle biopsies were combined to assess postprandial plasma glucose kinetics and intramuscular signaling, respectively.

Participants

Twelve healthy and physically active men (self-reported as regular exercisers engaging in at least 30 min of exercise a minimum of 3 times per week) were recruited from Bath and North East Somerset, between May and November 2015. Participant characteristics are shown in **Table 1**. Exclusion criteria included any history of metabolic disease, or condition that may have posed undue personal risk to the participant or introduced bias to the study.

Preliminary Testing

Participants were asked to refrain from strenuous physical activity for 24 h prior to preliminary testing, but were asked to otherwise maintain their normal physical activity behaviors. They abstained from alcoholic and caffeinated drinks for 24 h prior to this visit. Food intake ceased at 8 pm on the evening before testing and participants fasted overnight (minimum 12 h), consuming only water (*ad libitum*) during this period. In addition, they were asked to consume 568 mL of water at least 1 h prior to testing, and to void immediately prior to arriving at the laboratory. Upon arrival, the participant's stature was measured (Frankfurt plane) to the nearest 0.1 cm using a stadiometer (Seca Ltd, Birmingham, UK). Body mass was recorded to the nearest 0.1 kg (only light clothing permitted) using electronic weighing scales (BC543 Monitor, Tanita, Tokyo, Japan). A whole-body dual energy x-ray absorptiometry scan was completed to quantify fat and fat-free mass (DEXA; Discovery, Hologic, Bedford, UK).

Participants then performed an incremental cycling exercise test at a self-selected cadence on an electronically-braked ergometer (Excalibur Sport, Lode Lode® Groningen, Netherlands). They were permitted to adjust the saddle and handlebar heights to their preferred position, which were replicated for cycling during the exercise trials. The initial exercise intensity was 50 W and this was increased by 50 W every four min, for four stages. Thereafter, the intensity was increased by 20 W every min until volitional exhaustion. Heart rate (Polar Electro Oy, Kempele, Finland) and continuous breath-by-breath measurements were recorded throughout (TrueOne2400, ParvoMedics, Sandy, USA). Volume

and gas analyzers were calibrated with a 3 L calibration syringe (Hans Rudolph, Kansas City, USA) and a calibration gas (balance nitrogen mix; 16.04% O₂, 5.06% CO₂; BOC Industrial Gases, Linde AG, Munich, Germany), respectively. Peak power output (PPO) was calculated as the work rate of the last completed stage, plus the fraction of time spent in the final non-completed stage, multiplied by the work rate increment. Peak oxygen uptake ($\dot{V}O_2$ peak) was calculated as the highest average $\dot{V}O_2$ over a rolling 30-s period.

Main Trials

Participants refrained from strenuous physical activity, alcohol and caffeine for 24 h before all trials. They recorded the composition of their evening meal on the day before the first main trial and replicated this meal for subsequent trials, in accordance with procedures for standardizing postprandial glucose tolerance testing (10). This pre-trial standardisation protocol has been previously shown to be effective at producing overnight-fasted muscle glycogen concentrations, liver glycogen concentrations and intramyocellular lipid that are standardised across multiple trial days, in a similar population (21). To help ensure physical activity standardization, participants completed a physical activity diary and wore a physical activity monitor (ActiheartTM; Cambridge Neurotechnology, Papworth, UK) for 24 h before all trials (pre-trial 24-h physical activity energy expenditure [(mean \pm standard deviation) 988 \pm 500 kcal on BR, 1022 \pm 521 kcal on BE, 992 \pm 313 kcal on FE; all $p > 0.05$; $n = 9$].

Participants arrived at the laboratory at 0800 ± 1 h following a 12 to 14-h overnight fast and this arrival time was replicated for the subsequent trials. They were asked to void and all further urine samples were collected for the remainder of the trial to allow for urinary nitrogen excretion to be estimated from urine urea concentrations. Participants then placed their dominant hand into a heated-air box (Mass Spectrometry Facility; The University of Vermont & University of Vermont Medical Center, Burlington, USA) set to 55°C . After 20 min of rest, an intravenous catheter (BD Venflon Pro, BD, Helsingborg, Sweden) was fitted into a heated dorsal hand vein (retrograde) and a 10-mL baseline blood sample was drawn, before a 5-min expired gas sample was collected. On the first main trial for each participant (see **Figure 1**), a baseline muscle sample was taken from the *vastus lateralis* to allow for an assessment of the pathways involved in exercise and insulin signaling in muscle [5' AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), protein kinase B (herein referred to as Akt2), and Akt substrate of 160 kDa (AS160)].

In BE and BR, a porridge breakfast was then consumed within 10 min (3 h pre-OGTT) and in FE participants were allowed water only. The breakfast was 72 g of instant refined oats (Oatso Simple Golden Syrup, Quaker Oats) and 360 mL of semi-skimmed milk (Tesco), providing 431 kcal of energy ([1803 kJ]; 65 g CHO, 11 g FAT, 19 g PRO). The breakfast was high-carbohydrate (57% of energy intake) and high glycemic-index [oatmeal, made from mix (Quaker Oats) has a glycemic index of 69 (17)], as is commonly consumed in developed countries. Due to the co-ingestion of milk, this breakfast would produce a high

insulinemic response (38, 46). Breakfast consumption (or omission on FE) was followed by 2 h of rest, where participants remained in a semi-recumbent position, completing resting activities (e.g. watching television), with expired gas samples collected every 60 min. After 1 h 40 min of rest, (1 h 20 min pre-OGTT) a catheter was inserted into an antecubital vein (the contralateral arm to the one used for blood sampling). A primed infusion of [6,6-²H₂]glucose was initiated and continued for the remaining within-lab component of the trial (Cambridge Isotope Laboratories, MA, USA; prime: 13.5 $\mu\text{mol.kg}^{-1}$; infusion: 0.35 $\mu\text{mol.kg}^{-1}.\text{min}^{-1}$). After 20 min (60 min pre-OGTT), and on BE and FE only, participants began 60 min of cycling at 50% PPO on an ergometer (Lode Corival, Lode B.V, Groningen, Netherlands). The cadence was self-selected (replicated for both exercise trials) and the power output was monitored via a computerized system. In BR, participants remained rested in the semi-recumbent position during this period. Expired gas samples were collected every 15 min and blood samples were collected at 40 and 50 min of exercise (20 and 10 min pre-OGTT). Immediately post-exercise (or post-rest in BR) a muscle sample was taken from the *vastus lateralis*. Then a 2-h OGTT was completed, with arterialized blood sampled at 10-min intervals and expired gas sampled every 60 min. The OGTT was 73 g of glucose (81 g of dextrose monohydrate when corrected for water content; Myprotein, Northwich, UK) and 2 g of [U-¹³C]glucose (99%; Cambridge Isotope Laboratories, MA, USA), to allow the rate of appearance of the orally ingested glucose (Ra_{OGTT}) to be assessed. A final muscle sample was taken post-OGTT (OGTT 120 min).

Tracer approach, blood sampling and analysis

A dual-tracer approach was employed, where the tracer infusion rate was doubled during exercise (on BE and FE) to account for an expected increase in endogenous glucose production (1) and reduced to 80% of baseline at OGTT 20 min (all trials) to account for an expected suppression of endogenous glucose production after oral glucose ingestion (9). This approach reduces changes in the tracer-to-tracee ratio, thereby permitting more accurate estimations of glucose kinetics(4). Arterialized blood was sampled from a heated dorsal hand vein at baseline, at 60-min intervals during the initial 2-h rest period, at 40 and 50 min of the exercise period (or post-rest in BR) and at 10-min intervals during the OGTT. Whole blood was dispensed into ethylenediaminetetraacetic acid-coated tubes (BD, Oxford, UK) which were first centrifuged (4 °C and 3500 g) for 10-min (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK) to obtain plasma. The plasma was then dispensed into 0.5 mL aliquots and immediately frozen at -20 °C, before longer-term storage at -80 °C.

Plasma glucose (intra-assay coefficient of variation [CV], 3.2%; inter-assay CV 3.8%), lactate (intra-assay CV, 1.0%; inter-assay CV 4.8%), and triglyceride (intra-assay CV, 1.4%; inter-assay CV 4.0%) concentrations were measured using an automated analyzer (Daytona; Randox Lab, Crumlin, UK) as per the manufacturer's instructions. Plasma insulin concentrations were measured using a commercially available ELISA kit (Mercodia AB, Uppsala, Sweden; intra-assay CV, 5.7%; inter-assay CV 9.9%). Plasma intestinal fatty acid binding

protein (I-FABP) concentrations were measured as a marker of intestinal cell damage using a commercially available ELISA kit (Hycult Biotech; intra-assay CV, 6.0%). Plasma NEFA concentrations were determined using an enzymatic colorimetric assay (WAKO Diagnostics; intra-assay CV, 8.9%; inter-assay CV 10.4%). For all of these analyses, all plasma samples were analyzed in batch after all sample collection was completed, and for a given participant all samples (from the three trials) were run on the same plate.

Plasma [U-¹³C]glucose and [²H₂]glucose enrichments were determined by gas chromatography-mass spectrometry (GC-MS: GC, Agilent 6890N; MS, Agilent 5973N; Agilent Technologies, Stockport, UK). Plasma glucose was extracted using methanol-chloroform and hydrochloric acid, dried under nitrogen gas, and then derivatised using the heptofluorobutyric acid method as previously described(30). The glucose derivative was acquired by selected ion monitoring at mass-to-charge ratios (*m/z*) 519, 521 and 525 for [¹²C], [6,6-²H₂]- and [U-¹³C]-glucose, respectively. Glucose enrichments of [¹³C] and [²H₂] in plasma were determined using standard curves for [¹³C] and [²H₂] glucose, and enrichments were expressed relative to those at 519 (M+0). The baseline sample was used for every trial to account for background isotopic plasma enrichments. To reduce any impact of analytical variability on calculations of glucose kinetics, glucose and enrichment data were curve fitted as previously described (63).

Muscle sampling and analysis

Muscle samples were collected from the *vastus lateralis* under local anesthesia (~5 mL of 1% lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK). Samples were taken from a 3-5-mm incision at the anterior aspect of the thigh with a 5-mm Bergstrom biopsy needle technique adapted for suction(57). Samples were immediately extracted from the needle and frozen in liquid nitrogen, before longer-term storage at -80 °C. The order of dominant or non-dominant leg was counterbalanced across trials for the OGTT 0 min and the 120 min samples. Samples were taken from separate skin incision sites, with these > 2 cm proximal to any previous incision on the same leg (59). For the OGTT 0 min sample (post exercise [or post-rest in BR]) the incision was made prior to cycling (BE and FE) and closed with Steristrips, to allow for an immediate sample to be taken post-exercise.

Frozen wet tissue (20-30 mg) was freeze-dried, powdered, and dissected free of visible blood and connective tissue and added to ice cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Sodium deoxycholate; 0.1% SDS and 0.1% NP-40] with a protease (Thermo Scientific) and phosphatase inhibitor cocktail (Millipore). Samples were homogenized with a dounce homogenizer (~ 40 passes), incubated for 60 min at 4 °C with rotation, and centrifuged for 10 min (4 °C and 20,000 g). The protein content of the resultant supernatant was measured via a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). For Western blots, an equal amount of protein (40 µg) was loaded per lane for each sample and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-glycine SDS–polyacrylamide gels (7% for

298 p-AMPK^{Thr172}, total AMPK, p-ACC^{Ser79} and total ACC, 10% for p-Akt^{Ser473} and
299 total Akt2 and 8% for p-Akt^{Thr308}, p-AS160^{Thr642} and total AS160). Gels were
300 electro-blotted using a semi-dry transfer onto a nitrocellulose membrane.
301 Membranes were washed in Tris-buffered saline (0.09% NaCl, 100 mM Tris–
302 HCl pH 7.4) with 0.1% Tween 20 (TBS-T) and incubated for 30-min in a
303 blocking solution (5% non-fat dry milk in TBS-T; Marvel, Premier International
304 Foods Ltd, UK). The membranes were incubated overnight at 4 °C with primary
305 antibodies against p-AMPK^{Thr172}, p-ACC^{Ser79}, p-Akt^{Ser473}, p-Akt^{Thr308} and p-
306 AS160^{Thr642} (Cell Signaling Technologies, USA). In the morning, membranes
307 were washed in TBS-T and incubated with a 1:4000 dilution of anti-species IgG
308 horseradish peroxidase-conjugated secondary antibodies made up in the
309 aforementioned blocking solution. After further washes, membranes were
310 incubated in an enhanced chemiluminescence (ECL) reagent and visualized
311 using a chemiluminescent imager (EpiChemi II Darkroom, UVP, Upland, USA).
312 Nitrocellulose membranes were then incubated for 30-min at 50 °C in a
313 stripping solution [62.5 mM Tris pH 6.7, 2%SDS, 100 mM 2-mercaptoethanol],
314 before re-blotting for total AMPK, total ACC, total Akt2 (Cell Signaling
315 Technologies, USA) and total AS160 (Merck-Millipore, UK). For these
316 analyses, all samples from each participant (all three trials) were run on the
317 same gel. Band densities were quantified using VisionWorksLS Image
318 Acquisition and Analysis Software for Windows (UVP, Upland, USA). For all of
319 the signalling molecules reported in this experiment a ratio of phosphorylated to
320 total protein was calculated and the results were expressed relative to the
321 baseline sample.

Substrate utilization

Expired gas samples were collected at baseline, during the initial 2-h rest period and the OGTT at 60-min intervals (for 5 min), and at 15-min intervals (for 1 min) during the exercise period (or rest in BR). For all samples, participants were provided with the mouthpiece 1 min before gas collections for a stabilization period. Samples were collected in 200 L Douglas bags (Hans Rudolph, Kansas City, USA) through falconia tubing (Baxter, Woodhouse and Taylor Ltd, Macclesfield, UK). Concurrent measures of inspired air were made to correct for changes in ambient O₂ and CO₂ concentrations(7). Expired O₂ and CO₂ concentrations were measured in a known volume of each sample, using paramagnetic and infrared transducers respectively (Mini HF 5200, Servomex Group Ltd., Crowborough, East Sussex, UK). The sensor was calibrated using concentrations of low (99.998% Nitrogen, 0% O₂ and CO₂) and high (balance nitrogen mix, 16.04% O₂, 5.06% CO₂) calibration gases (both BOC Industrial Gases, Linde AG, Munich, Germany). Urinary nitrogen excretion was estimated from urine urea concentrations, which were measured on an automated analyzer (Daytona; Randox Lab, Crumlin, UK), to allow for protein oxidation to be accounted for in calculations of substrate utilization rates.

Calculations and statistical analysis

A sample size estimation was completed *a priori* with the total rate of plasma glucose appearance as the primary outcome measure. Rose *et al.* (54) reported a difference in the total plasma glucose appearance of (mean \pm SD) 1600 \pm

1300 $\mu\text{mol}\cdot\text{kg}^{-1}$ during an OGTT after rest *versus* after fasted exercise. Using this effect size, and an alpha level of 0.05, we calculated that 12 participants were required for an 80% probability of statistically detecting an effect in the Ra_{TOTAL} using a crossover design with three trials and a two-tailed, one-way ANOVA.

The total and incremental area underneath the concentration-time curve (AUC or iAUC, respectively) for each plasma metabolites or hormones was calculated using the trapezoid rule. The AUC or iAUC for each plasma metabolite or hormone was then divided by either the duration of the total within-lab period (300-min) or the OGTT observation period (120-min), as appropriate, to provide a time-averaged value ($\text{mmol}\cdot\text{L}^{-1}$), which are used as summary measures. Plasma glucose and insulin concentrations during the OGTT were used to estimate insulin sensitivity (ISI) according to the equation of Matsuda (41): [FPG and FPI are fasting plasma glucose and insulin concentrations, and MPG and MPI are mean plasma glucose and insulin concentrations in the OGTT (41)]:

$$\text{ISI}_{\text{MATSUDA}}(\text{au}) = \frac{10,000}{\sqrt{\text{FPG}(\text{mgdL}^{-1}) \cdot \text{FPI}(\text{mIU mL}^{-1}) \cdot \text{MPG}(\text{mgdL}^{-1}) \cdot \text{MPI}(\text{mIU mL}^{-1})}}$$

Plasma glucose kinetics were determined using Radziuk's two-compartment non-steady state model (50, 51) and SAMM II software (SAAM II v2.3, The Epilson Group, Charlottesville, VA, USA). This model reduces errors in estimations of glucose kinetics that are apparent when using Steele's (56) one

compartment model (53). The total rate of plasma glucose appearance (Ra_{TOTAL}) and glucose disappearance (Rd) were calculated as follows:

Equation 1:

$$Ra_{TOTAL}(t) = \frac{F}{E_1(t)} - \frac{V_1 \cdot G(t)}{E_1(t)} \cdot \dot{E}_1(t) + k_{12} \left(\frac{q_2^{iv}(t)}{E_1(t)} - Q_2(t) \right)$$

Equation 2:

$$Rd(t) = Ra_{TOTAL} - V_1 \cdot \dot{G}(t) - k_{21} \cdot V_1 \cdot G(t) + k_{12} \cdot Q_2(t)$$

Where F is the $[6,6-^2H_2]$ infusion, V_1 is the glucose volume of distribution [4% of body mass (kg)], $E_1(t)$ the $[^2H_2]$ plasma glucose enrichment (mole percent excess) at time t , $\dot{E}_1(t)$ the change in E over time [derivate of E], $G(t)$ the plasma glucose concentrations at time t , $\dot{G}(t)$ the change in G over time [derivate of G], k_{12} and k_{21} are fixed rate constants between the peripheral and the accessible compartments (0.05 min^{-1} and 0.07 min^{-1} respectively) and q_2^{iv} and Q_2 are the amounts of the tracer $[^2H_2]$ and tracee in the peripheral compartment respectively, evaluated by integrating the two-compartment model.

The $[U-^{13}C]$ enrichment of the orally ingested glucose and the Ra_{TOTAL} (from *Equation 1*) were used to calculate the plasma rate of appearance of glucose from the OGTT (Ra_{OGTT}). In these equations, r_1 is the ratio of the infusion $[^2H_2]$ and oral $[U-^{13}C]$ glucose tracer concentrations in plasma, $\dot{r}(t)$ is the change in r over time [derivate of r], g is the $[U-^{13}C]$ glucose tracer in plasma, q_o^{iv} is the

amount of the [U-¹³C] tracer in the peripheral compartment (by integrating the two-compartment model), and E_{OGTT} is the [U-¹³C] enrichment of the OGTT.

Equation 3:

$$ra^O(t) = Ra_{TOTAL}(t) \frac{F}{r_1(t)} - \frac{V_1 \cdot g(t)}{r_1(t)} \cdot \dot{r}_1(t) + k_{12} \left[\frac{q_2^{iv}(t)}{r_1(t)} - q_2^O(t) \right]$$

Equation 4:

$$Ra_{OGTT}(t) = ra^O(t) \left[\frac{1}{E_{OGTT}} \right]$$

The metabolic clearance rate was calculated as the Rd divided by the plasma glucose concentrations for a given time point (G₁)

Equation 5:

$$\text{Metabolic Clearance Rate} = Rd(t) / G_1$$

Rates of whole-body fat and carbohydrate utilization were calculated using the expired gas samples and stoichiometric equations (31). Adjustments were made to account for the contribution made by the oxidation of protein (estimated via urinary urea nitrogen). Plasma glucose utilization was assumed to be equivalent to the plasma glucose rate of disappearance (Rd) as has been confirmed previously (32). Muscle glycogen utilization during exercise (BE and FE only) was calculated as total carbohydrate utilization during exercise minus plasma glucose utilization during exercise. Due to these methods, this estimate of muscle glycogen utilization will include the utilization of other non-glucose carbohydrates (e.g. lactate). Both the production and utilization of ketone

bodies can influence the respiratory exchange ratio and therefore theoretically complicate the estimates of carbohydrate oxidation during exercise. However, during short-duration, moderate intensity exercise this effect is negligible (31). Within-lab, energy expenditure was determined assuming that lipids, glucose and glycogen give 40.81, 15.64 and 17.36 kJ·g⁻¹ respectively (31).

One-way, repeated measures ANOVA were used to assess differences between trials at baseline and for summary measures (e.g. AUCs). If multiple comparisons were necessary, two-way repeated measures ANOVAs (time x trial) was used to identify differences between trials. Degrees of freedom for *F* values were Greenhouse-Geisser corrected for epsilon < 0.75, with Huynh-Feldt corrections used for less severe asphericity. If time x trial interaction effects were identified, multiple paired *t*-tests were used to locate variance, with Holm-Bonferroni step-wise adjustments to control for inflated type I errors. Pearson *r* and Spearman *R* were used to explore correlations between variables display normal and non-normal distribution, respectively. Unless otherwise stated, data in text, figures and tables are means ± 95% confidence intervals, which were normalized by removing between-subject variance (presented as 95% nCI)(39). All statistical analyses were completed using IBM SPSS statistics version 22 for windows (IBM, New York, USA), with the exception of the Holm-Bonferroni step-wise adjustments and the calculation of normalized confidence intervals which were completed using Microsoft Excel [2013]. Graph Pad Prism 7 software (La Jolla, CA, USA) was used for preparation of the manuscript figures. A complete set of muscle samples was only obtained from nine

participants. Due to cannulation difficulties, for one participant's BR trial the last blood sample obtained was at OGTT 70 min and for a different participant's BE trial the last sample was at OGTT 60 min. For these trials (2 of 36) the group average was used for the missing data. Sensitivity analysis was completed for all measures involving blood samples and including/excluding these two participants did not influence any of the primary outcome measures. For clarity, the *n* is presented in all figure and table legends.

RESULTS

Plasma glucose kinetics

The plasma glucose disappearance rate (R_d) displayed a time x trial interaction ($F = 3.123$, $p = 0.05$), whereby plasma glucose R_d was higher during exercise *versus* rest (**Figure 2A**). Compared to extended overnight fasting, breakfast ingestion prior to exercise further increased the plasma glucose R_d during and after exercise (i.e. during the OGTT; **Figure 2A**). A main effect of trial was detected for the plasma glucose R_d during the OGTT ($F = 7.079$, $p = 0.01$), whereby the R_d was $45 \text{ g} \cdot 120 \text{ min}^{-1}$ in BR [95% nCI: 36 to $62 \text{ g} \cdot 120 \text{ min}^{-1}$] *versus* $73 \text{ g} \cdot 120 \text{ min}^{-1}$ in BE ([95% nCI: 55 to $90 \text{ g} \cdot 120 \text{ min}^{-1}$; $p = 0.09$ *versus* BR) and $44 \text{ g} \cdot 120 \text{ min}^{-1}$ in FE [95% nCI: 35 to $53 \text{ g} \cdot 120 \text{ min}^{-1}$; $p = 0.01$ *versus* BE). Metabolic clearance rates showed a main effect of trial, with the highest rates also apparent in BE (**Figure 2B**; $F = 7.849$, $p < 0.01$ *versus* BR and FE).

A main effect of trial was detected for $R_{a\text{TOTAL}}$ during the OGTT ($[g \cdot 120 \text{ min}^{-1}]$; $F = 3.915$, $p = 0.05$) which was highest in BE (**Figure 2C**). However, after post-hoc adjustment the difference between trials was less apparent ($p = 0.19$ for BE *versus* BR and $p = 0.09$ for BE *versus* FE). A similar pattern was observed for the rate of appearance of glucose from the OGTT in plasma ($R_{a\text{OGTT}}$) and a trial x time interaction was detected (**Figure 2D**; $F = 3.134$, $p = 0.04$). A main effect of trial was detected for the total $R_{a\text{OGTT}}$ ($F = 5.915$, $p = 0.02$), which was $49 \text{ g} \cdot 120 \text{ min}^{-1}$ in BE [95% nCI: 44 to $53 \text{ g} \cdot 120 \text{ min}^{-1}$] (65% of the OGTT [59 to 71%]) *versus* $42 \text{ g} \cdot 120 \text{ min}^{-1}$ in BR [95% nCI: 36 to $46 \text{ g} \cdot 120 \text{ min}^{-1}$] (56% of the OGTT [50 to 62%]; $p = 0.11$ *versus* BE) and $41 \text{ g} \cdot 120 \text{ min}^{-1}$ in FE [95% nCI: 35

to 47 g] (55% of the OGTT [49 to 61%] $p = 0.06$ *versus* BE). The plasma enrichment of [$^2\text{H}_2$] - and [^{13}C]- glucose are shown in **Figures 2E** and **2F**, respectively.

Plasma glucose concentrations

No difference between trials was detected for plasma glucose concentrations at baseline (Figure 3A; $p > 0.05$). Thereafter, a trial x time interaction was apparent ($F = 2.957$, $p = 0.01$). During the exercise period (rest in BR), plasma glucose concentrations were higher in BR *versus* BE at 40 min, and in BR *versus* FE at 50 min (both $p < 0.05$). At OGTT 0 min, glucose concentrations were higher in BR *versus* BE, and during the OGTT they were initially higher in BR *versus* BE and in BR *versus* FE (all $p < 0.05$), but no further differences were then detected (Figure 3A). Peak plasma glucose concentrations were higher in BR *versus* BE ($p = 0.03$), but not different in BE *versus* FE (Table 2; $p > 0.05$). A main effect of trial was detected for the within-lab (300-min) glucose AUC which was higher in BR *versus* BE (Table 2; $p = 0.05$). However, no main effect of trial was detected for the OGTT (120-min) iAUC (Figure 4A; $F = 2.524$, $p = 0.11$).

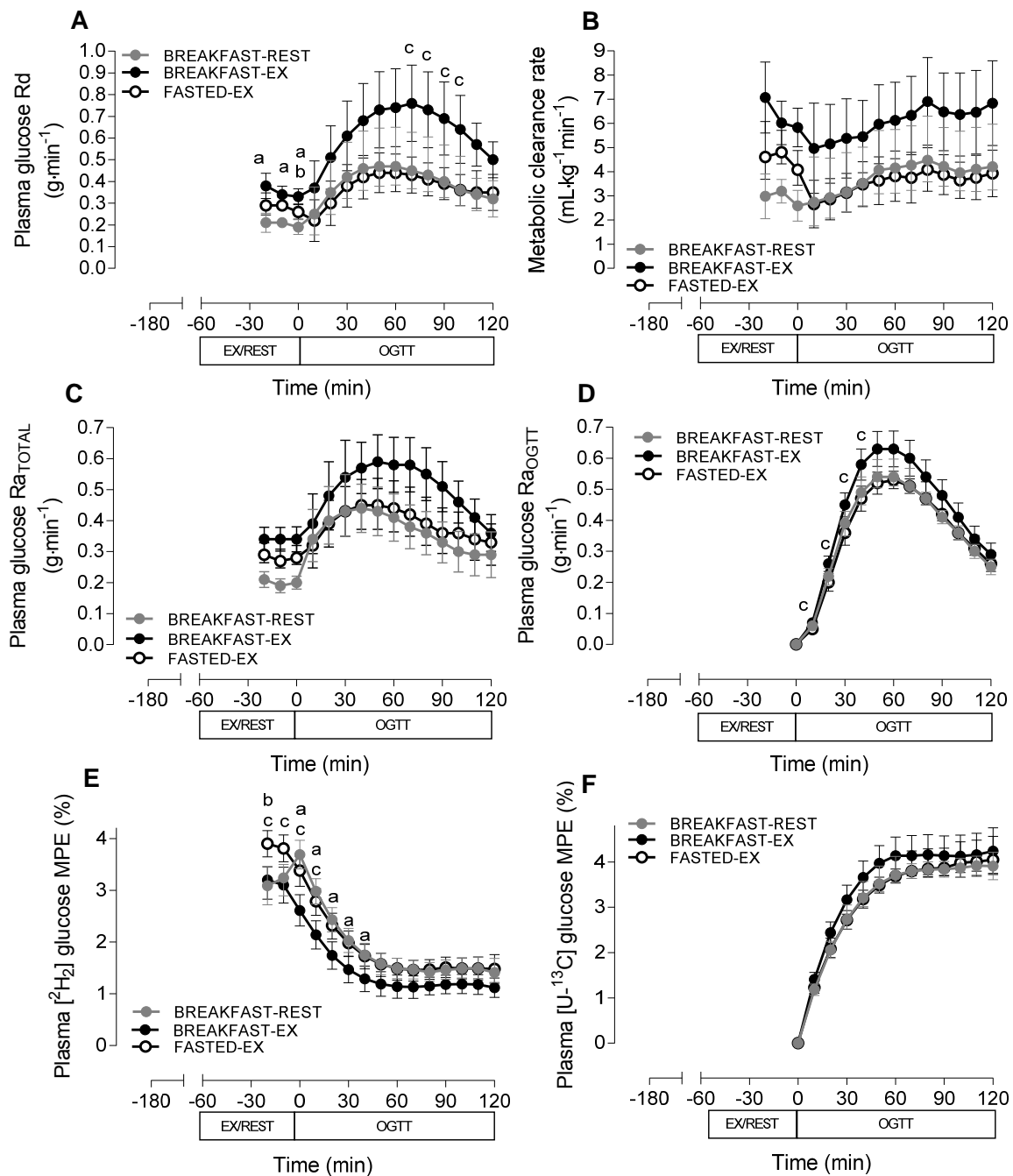


Figure 2. The plasma glucose disposal rate (Rd) (A), metabolic clearance rate (B), the total rate of plasma glucose appearance (Ra_{TOTAL}) (C), the rate of appearance of glucose in plasma from the oral glucose tolerance test (Ra_{OGTT}) (D), and the plasma enrichments of $[\text{H}_2]$ -glucose (E) and $[\text{C}]$ -glucose (F) before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means \pm normalized 95% confidence intervals. $n = 12$ healthy men. a = difference between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus* fasted exercise and c = breakfast exercise *versus* fasted exercise with $p < 0.05$.

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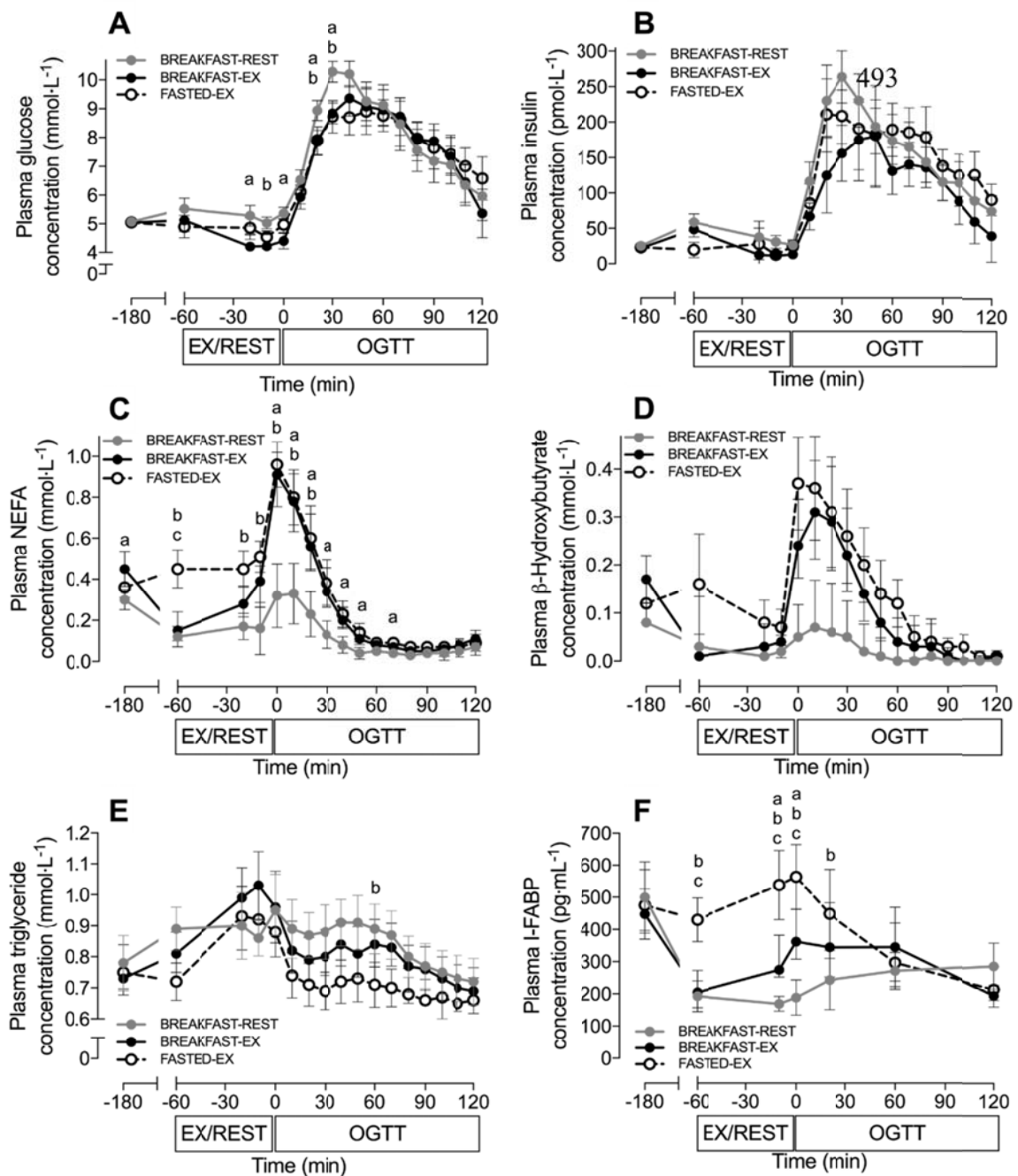


Figure 3. Plasma glucose (**A**), plasma insulin (**B**), plasma non-esterified fatty acids (NEFA; **C**), plasma β -hydroxybutyrate (**D**), plasma triglyceride (**E**), and plasma intestinal fatty acid binding protein (I-FABP; **F**) concentrations before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means \pm normalized 95% confidence intervals. $n = 12$ healthy men. a = difference between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus* fasted exercise and c = breakfast exercise *versus* fasted exercise with $p < 0.05$.

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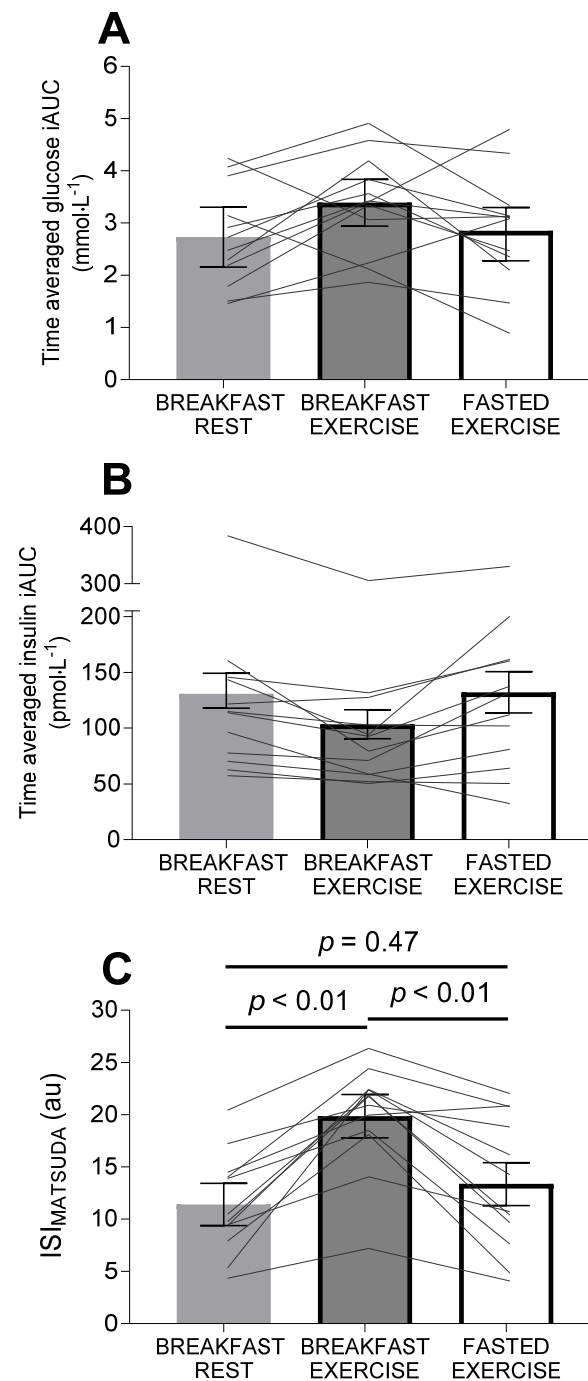


Figure 4. The time-averaged (120-min) plasma glucose (**A**) and plasma insulin (**B**) incremental area under the curves (iAUC) and the Matsuda insulin sensitivity index (**C**; ISI_{MATSUDA}) for an oral glucose tolerance test conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means \pm normalized 95% confidence intervals, with individual data shown as grey lines. $n = 12$ healthy men.

Plasma insulin concentrations

At baseline, there was no difference between trials for plasma insulin concentrations (**Figure 3B**; $p > 0.05$). Main effects of time ($F = 4.351$, $p < 0.01$) and trial ($F = 7.986$, $p < 0.01$) were detected, but there was no trial x time interaction effect ($F = 2.395$, $p = 0.07$). Peak (and time to peak) plasma insulin concentrations are shown in **Table 2**. A main effect of trial was detected for the within-lab (300-min) insulin AUC which was higher in BR *versus* BE ($p < 0.01$), but not different in BE *versus* FE (**Table 2**; $p = 0.10$). A main effect of trial was apparent for the insulin OGTT iAUC [**Figure 4B**; (120-min) $F = 5.132$, $p = 0.02$] which was lower in BE *versus* BR [by $27.34 \text{ pmol}\cdot\text{L}^{-1}$ (95% nCI: 12.10 to $45.80 \text{ pmol}\cdot\text{L}^{-1}$); $p = 0.02$] and lower in BE *versus* FE [by $28.67 \text{ pmol}\cdot\text{L}^{-1}$ (95% nCI: 10.21 to $47.12 \text{ pmol}\cdot\text{L}^{-1}$); $p = 0.04$]. There was a main effect of trial for the ISI_{MATSUDA} insulin sensitivity index (**Figure 4C**; $F = 22.790$, $p < 0.01$), which was higher in BE *versus* BR [by 8.45 au, (95% nCI: 6.42 to 10.47 au); $p < 0.01$] and in BE *versus* FE [by 6.49 au (95% nCI: 2.93 to 8.51 au); $p < 0.01$].

Plasma non-esterified fatty acid (NEFA) concentrations

A main effect of trial ($F = 4.314$, $p = 0.04$) was detected for plasma NEFA at baseline, with concentrations of $0.30 \text{ mmol}\cdot\text{L}^{-1}$ in BR (95% nCI: 0.25 to $0.35 \text{ mmol}\cdot\text{L}^{-1}$), $0.45 \text{ mmol}\cdot\text{L}^{-1}$ in BE (95% nCI: 0.36 to $0.53 \text{ mmol}\cdot\text{L}^{-1}$; $p = 0.03$ BE *versus* BR) and $0.36 \text{ mmol}\cdot\text{L}^{-1}$ in FE (95% nCI: 0.31 to $0.44 \text{ mmol}\cdot\text{L}^{-1}$; $p = 0.12$ FE *versus* BR and BE). Thereafter, a time x trial interaction effect was apparent (**Figure 3C**; $F = 11.438$, $p < 0.01$), where plasma NEFA concentrations were lowered by breakfast consumption in BR and BE, and remained lower during

the exercise in BE *versus* FE, before increasing during the initial OGTT period in BE and FE *versus* BR. A main effect of trial was detected for the total within-lab plasma NEFA (300-min) AUC and the NEFA OGTT (120-min) AUC which in both instances was lower in BR *versus* BE and FE (**Table 2**; all $p < 0.01$).

Other plasma metabolites

No differences were detected between trials at baseline for plasma β -hydroxybuturate concentrations (**Figure 3D**; $p > 0.05$). Thereafter, a time x trial interaction effect was apparent ($F = 6.310$, $p < 0.01$) where concentrations were lowered by breakfast in BR and BE. Plasma β -hydroxybuturate concentrations remained lower during exercise in BE *versus* FE, but increased during the OGTT with BE and FE *versus* BR. However, with post-hoc adjustment, the differences between trials for plasma β -hydroxybuturate concentrations became less clear (all $p > 0.05$). The within-lab (300-min) β -hydroxybuturate AUC was lower with BR *versus* BE ($p = 0.03$), but did not differ in BE and FE (**Table 2**; $p = 0.35$). No baseline differences were detected for triglyceride concentrations (**Figure 3E**, $p > 0.05$) but a time x trial interaction effect was apparent ($F = 3.994$, $p < 0.01$). There was an effect of trial for the within-lab (300-min) and OGTT (120-min) triglyceride AUC, which tended to be lower in FE *versus* BE, but with post-hoc adjustment this difference between trials was less clear (**Table 2**; $p > 0.05$). Plasma lactate concentrations at baseline were not different across trials ($p > 0.05$) but were lower in BR *versus* BE and FE in the exercise period (rest in BR) and at OGTT 0 min, but were then higher in BR *versus* BE during the OGTT (time x trial; $F = 20.305$, $p < 0.01$). No effect of trial was

detected for the total within-lab (300-min) lactate AUC, but a main effect of trial was detected for the lactate OGTT (120-min) AUC, which higher in BR *versus* BE (**Table 2**; $p < 0.01$).

Plasma intestinal fatty acid binding protein (I-FABP) concentrations

There was no difference between trials at baseline for plasma I-FABP concentrations (**Figure 3F**; $p > 0.05$), but these were lower after breakfast (time x trial interaction effect; $F = 6.844$, $p < 0.01$) in BR and BE *versus* FE (both $p < 0.05$). During and post-exercise (or rest in BR), I-FABP concentrations were lower in BR and BE *versus* FE and remained lower in BR *versus* FE until OGTT 20 min (all $p < 0.05$). The within-lab (300-min) I-FABP AUC was lower in BR and BE *versus* FE (**Table 2**; $p = 0.01$ and $p = 0.05$ respectively).

Activation of exercise and insulin signaling pathways in skeletal muscle

Time x trial interaction effects were apparent for AMPK^{Thr172} (ratio p-AMPK to total-AMPK) and ACC^{Ser79} (ratio pACC to total ACC) phosphorylation, if normalized to the baseline muscle sample (**Figure 5A**; $F = 5.154$, $p = 0.04$ and **Figure 5B**, $F = 5.881$, $p = 0.02$, respectively). Compared to the breakfast-rest trial (BR), skeletal muscle AMPK^{Thr172} phosphorylation was higher post-exercise (or post-rest in BR) in the breakfast and exercise (BE) trial [by 1.9 fold (95% nCI: 0.9 to 2.8 fold); $p = 0.04$] and was also higher in BE *versus* the fasted-exercise (FE) trial [by 1.0 fold (95% nCI: 0.2 to 2.0 fold); $p = 0.01$]. A similar pattern was apparent for ACC phosphorylation, which was higher post-exercise (or post-rest in BR) in BE *versus* BR [by 6.7 fold (95% nCI: 5.4 to 8.0 fold); $p =$

0.03] but did not differ between BE and FE ($p = 0.09$). By OGTT 120 min, ACC^{Ser79} and AMPK^{Thr172} phosphorylation had returned to baseline levels in all three trials (all $p > 0.05$). No time x trial interaction ($F = 2.110$, $p = 0.16$) nor a main effect of trial ($F = 0.098$, $p = 0.83$) was detected for Akt^{Ser473} (ratio p-Akt^{Ser473} to total-Akt2) phosphorylation (**Figure 6A**). A main effect of time ($F = 9.907$, $p = 0.01$) was observed, where Akt^{Ser473} phosphorylation was elevated at OGTT 120 min in all trials. Similarly no time x trial interaction ($F = 1.533$, $p = 0.25$) nor a main effect of trial ($F = 0.484$, $p = 0.56$) was detected for Akt^{Thr308} (ratio p-Akt^{Thr308} to total-Akt2) phosphorylation (**Figure 6B**). A main effect of time ($F = 10.598$, $p = 0.01$) was also detected for this phosphorylation site, whereby Akt^{Thr308} phosphorylation was elevated at OGTT 120 min in all trials. For AS160^{Thr642} phosphorylation (ratio p-AS160^{Thr642} to total-AS160), a time x trial interaction was detected (**Figure 6C**; $F = 4.430$, $p = 0.03$), whereby the AS160^{Thr642} phosphorylation was not different between BR and BE at any time, was higher pre-OGTT in BE compared to FE ($p = 0.04$), but was not different between BE and FE at 120-min post-OGTT ($p = 0.69$).

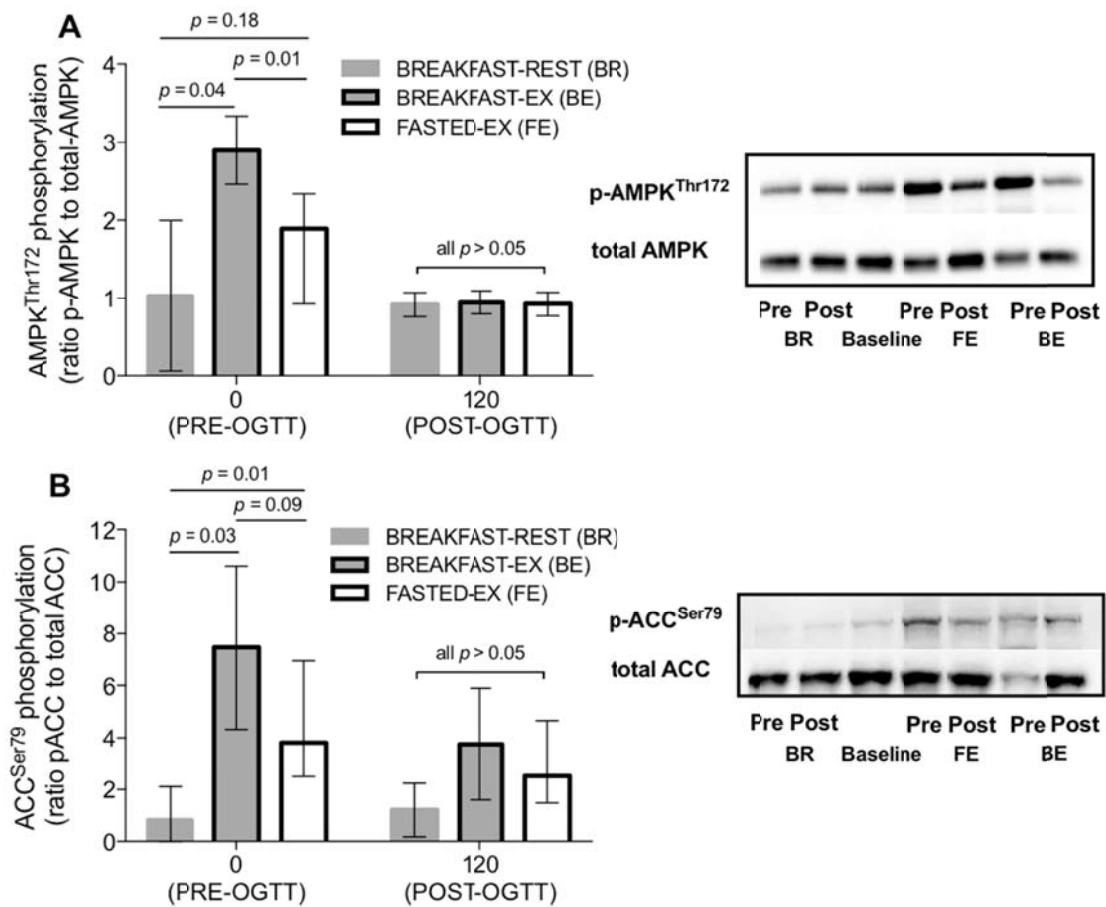


Figure 5. The phosphorylation of 5' AMP-activated protein kinase (**A**; **phospho** AMPK^{Thr172}, ratio p-AMPK to total-AMPK) and the phosphorylation of acetyl-CoA carboxylase (**B**; **phospho** ACC^{Ser79}, ratio p-ACC to total-ACC) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. **Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant).** Data are means \pm normalized 95% confidence intervals. $n = 9$ healthy men.

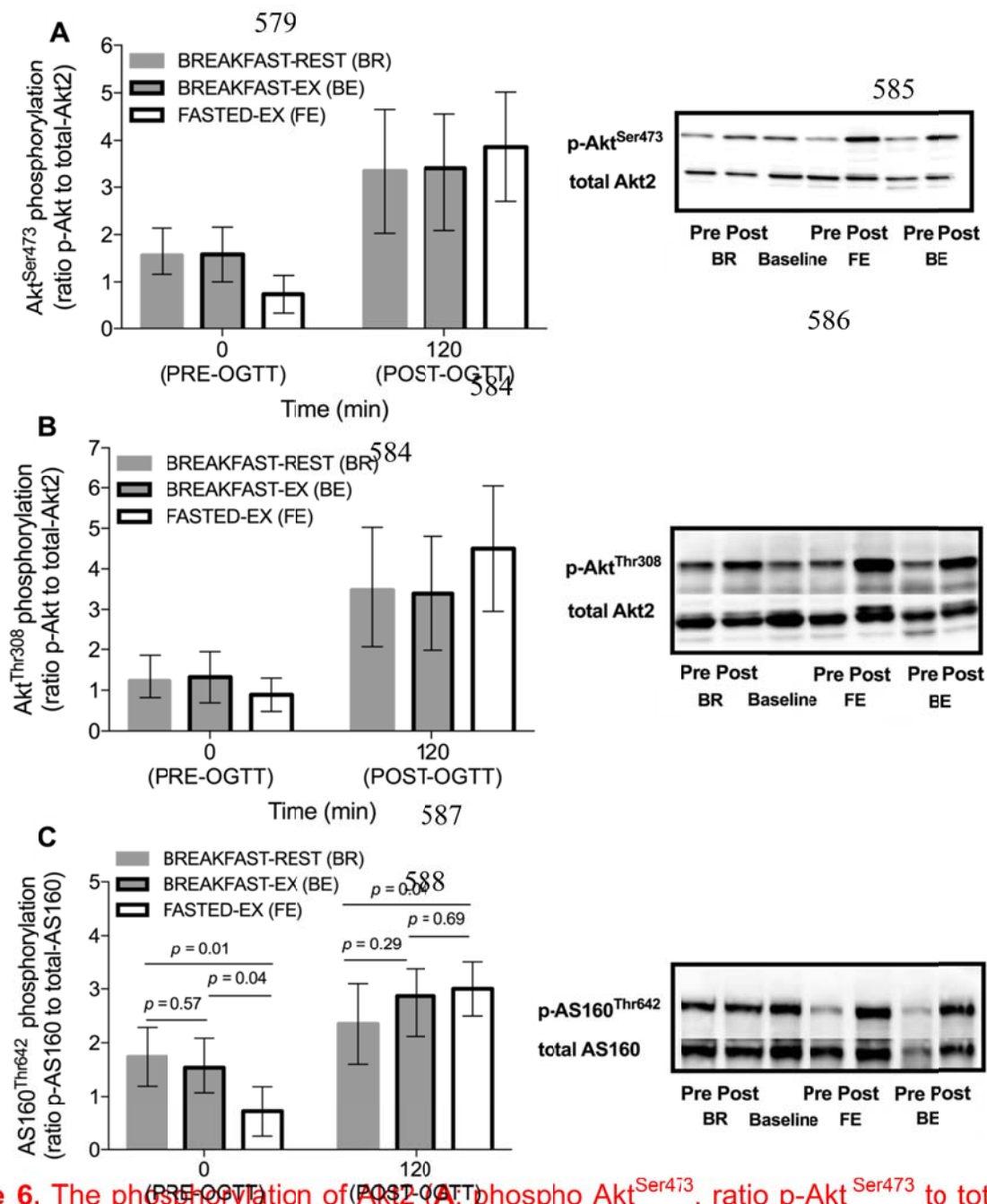


Figure 6. The phosphorylation of Akt2 (A; phospho Akt^{Ser473}, ratio p-Akt^{Ser473} to total-Akt2) and B; phospho Akt^{Thr308}, ratio p-Akt^{Thr308} to total-Akt2) and the phosphorylation of AS160 (C; AS160^{Thr642}, ratio p-AS160^{Thr642} to total-AS160) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means \pm normalized 95% confidence intervals. $n = 9$ healthy men.

Substrate utilization

Across the duration of the trial carbohydrate utilization was higher in BE *versus* BR (**Figure 7**; by 514 kcal, [95% nCI: 452 to 576 kcal] and higher in BE *versus* FE (by 124 kcal, [95% nCI: 18 to 230 kcal], both $p < 0.01$). This difference in carbohydrate utilization between BE and FE was derived from a higher utilization of plasma glucose *and* other carbohydrate sources (i.e. primarily muscle glycogen, but also plasma lactate) in BE ($p = 0.02$ and $p = 0.04$ respectively). Within-lab fat utilization did not differ between BR and BE ($p = 0.25$), but was higher in FE *versus* BE (by 138 kcal, [95% nCI: -6 to 224 kcal], $p = 0.03$). Muscle glycogen utilization during exercise ($\text{g} \cdot \text{kg body mass}^{-1}$) was positively correlated ($R = 0.64$, $p < 0.01$) with skeletal muscle ACC^{Ser79} phosphorylation (ratio p-ACC to total-ACC) after exercise conducted following breakfast consumption (BE) or extended overnight fasting (FE).

601

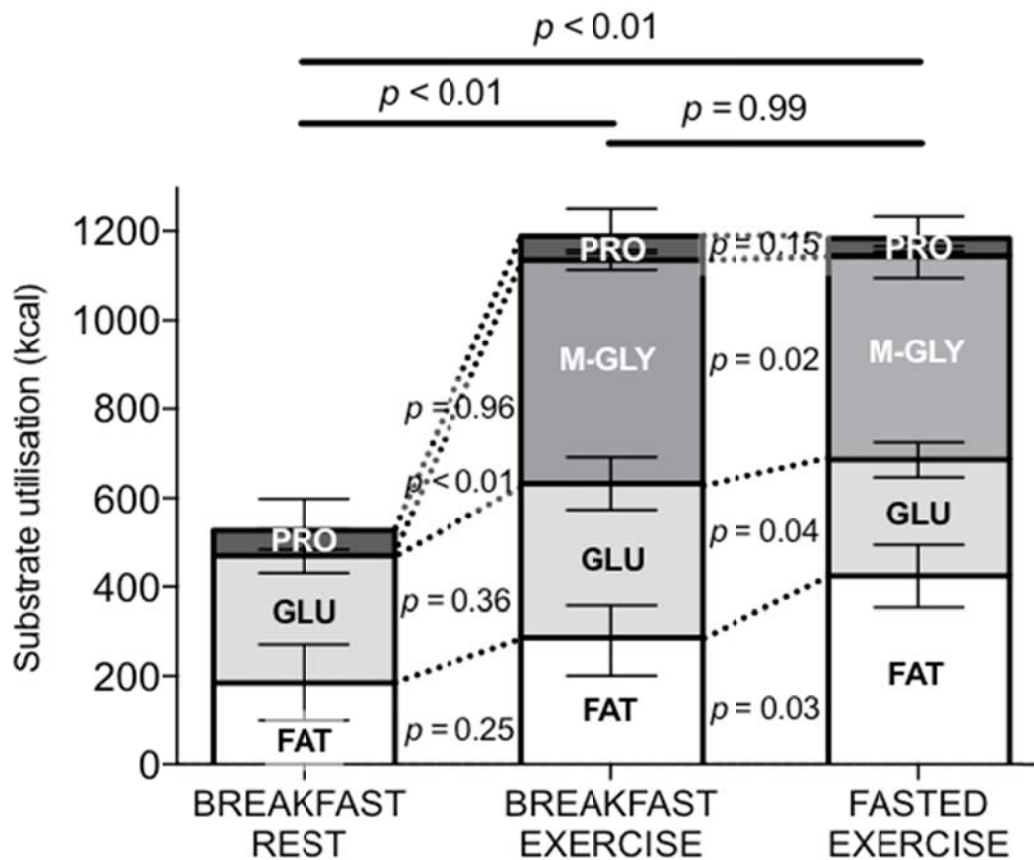


Figure 7. Substrate utilization after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Within-lab substrate utilization was calculated assuming that the plasma glucose disappearance rate was equivalent to plasma glucose utilization. Glycogen utilization during exercise (on breakfast exercise and fasted exercise only) was calculated as total carbohydrate utilization (from indirect calorimetry) minus plasma glucose utilization during exercise. This estimate of muscle glycogen utilization will therefore include the utilization of other non-glucose (e.g. lactate) carbohydrates. GLU = glucose; M-GLY = muscle glycogen; PRO = protein. Data are presented as means \pm normalized 95% confidence intervals. $n = 12$ healthy men.

602

DISCUSSION

This is the first study to assess the effect of pre-exercise feeding on postprandial plasma glucose kinetics after exercise. Our data demonstrate that pre-exercise feeding increases plasma glucose disposal during meals consumed after exercise, despite lower insulinemia in this condition. Characterizing glucose flux at meals is important because this determines postprandial glycemia; a predictor of cardiovascular disease risk (47, 48). Previous work has only studied postprandial glucose flux after fasted-state exercise. As most people consume food and perform exercise while still in a postprandial period from a prior meal (13, 23, 55) our results describe the physiological responses to feeding that are more readily applicable to scenarios that are representative of normal daily living. Moreover, our novel data demonstrate that metabolic responses to exercise conducted in an overnight fasted state and to meals that are consumed post-exercise cannot be easily extrapolated to conditions where breakfast has been consumed.

The disposal of plasma glucose (the disappearance rate) into skeletal muscle is elevated after exercise, via insulin-dependent and -independent pathways (25). We observed a higher postprandial plasma glucose disposal rate with breakfast *versus* fasting before exercise. The higher postprandial plasma glucose rate of disposal with pre-exercise breakfast ingestion was apparent despite lower insulinemia in the breakfast-exercise trial. At rest, breakfast consumption improves glucose tolerance and insulin sensitivity at subsequent meals [known as the “second-meal effect” (5, 20, 26)]. Mechanisms likely relate to delayed

gastric emptying (19), a potentiation of early phase insulinemia at the second meal (37) and enhanced glucose uptake into muscle due to increased GLUT4 trafficking (18). Our findings show that breakfast ingestion (*versus* fasting) prior to exercise enhances subsequent glucose disposal at post-exercise meals in the presence of lower insulinemia, suggesting that the second-meal effect is maintained even if exercise is performed between meals. Whilst the effects of pre-exercise feeding on the metabolic responses during subsequent exercise is well characterised (15, 43), our data therefore provide new insights regarding postprandial glucose metabolism after exercise in the fed *versus* fasted state.

Molecular insulin-signaling pathways are instrumental mediators of glucose disposal in response to exercise and/or nutrition (11). We therefore determined the activation status of key proteins involved in glucose uptake in skeletal muscle [the primary site of postprandial glucose disposal (14)]. Akt activation (Thr³⁰⁸ and Ser⁴⁷³ phosphorylation) two hours after OGTT began, was unaffected by prior exercise or prior breakfast ingestion. However, the timing of muscle sampling could be responsible for this result. It is possible that differences in Akt activation between trials may have been apparent earlier in the postprandial period, as peak Akt phosphorylation can be variable, occurring as early as 30 min after an OGTT (35). Thus, despite the lack of a measurable difference, we cannot rule out a role for insulin signaling in the glucose disposal responses we observed. Distal proteins within the insulin signalling pathway can be activated after exercise, without detectable differences in Akt phosphorylation (61). We therefore measured AS160^{Thr642} activation as this

phosphorylation site has been previously shown to be activated by both insulin *and* exercise stimulation (58). However, our data show that AS160^{Thr642} phosphorylation was not different two hours post-OGTT with breakfast *versus* fasting before exercise, providing further evidence that differences in early stages of the activation of the insulin signalling pathway were not responsible for the higher plasma glucose disposal rate we observed when breakfast was ingested before exercise.

AMPK activity also plays a key role in muscle glucose uptake and stimulate GLUT4 translocation (27). The greater post-exercise skeletal muscle AMPK activation with breakfast prior to exercise may have contributed to the higher glucose disposal rate in that trial. This AMPK response seems to be specific to skeletal muscle, as we have previously shown that post-exercise adipose tissue AMPK content is unaffected by pre-exercise feeding (12). The increase in skeletal muscle AMPK activity with pre-exercise feeding that we report in the present study may seem surprising given that the ingestion of large amounts (> 200 g) of carbohydrate before *and* during exercise can blunt AMPK signaling in muscle (3, 29). This blunting is partly because low muscle glycogen concentrations stimulate AMPK activity (42). The *modest amount* (65 g) of carbohydrate ingested by participants in our study may explain why we did not observe an elevated AMPK response in our fasted-exercise trial. For example, when smaller carbohydrate doses are ingested before and/or during exercise (~ 120 g or less) the exercise induced increase in the phosphorylation of AMPK and ACC is not always suppressed compared to when a placebo is ingested (2,

36, 58), although in one study this result was apparent despite a suppression of $\alpha 2$ -AMPK activity when carbohydrate was ingested (2).

The heightened AMPK response observed in the current study with breakfast before exercise may be explained by the *type* of carbohydrate ingested before exercise (high-glycemic index) in the breakfast-exercise trial, as this can stimulate muscle glycogen use during exercise, especially when no carbohydrate is ingested during the activity (62). Thus, the high-glycemic index breakfast with a modest carbohydrate content in the present study, may have stimulated muscle glycogen utilization during exercise without supplying sufficient carbohydrate to replace additional glycogen utilization, resulting in lower post-exercise muscle glycogen concentrations with breakfast *versus* fasting before exercise. This could explain the enhanced exercise-induced AMPK response if breakfast was consumed prior to exercise. However, we had insufficient tissue to measure muscle glycogen *concentrations* in all participants, and our data only provide rates of muscle glycogen *utilization* (which was higher with feeding *versus* fasting before exercise). Nevertheless, in the two participants for whom we had sufficient tissue to perform glycogen analyses, these data were in agreement with the tracer-derived calculations of muscle glycogen utilization (data not shown) and AMPK signaling assessed by western blotting. Consistent with this, the present data demonstrated that post-exercise ACC^{Ser79} phosphorylation – as a marker of activation of the AMPK pathway - positively correlated with muscle glycogen utilization during exercise. Thus, taken together our results suggest that the increased post-exercise plasma

glucose disposal rates with pre-exercise feeding *versus* fasting may have been mediated through enhanced AMPK signaling, which is itself a consequence of altered fuel use during exercise. AMPK signaling and resulting muscle glycogen concentrations following exercise after breakfast should now be explored.

The higher plasma glucose disposal rate (despite lower plasma insulin concentrations), that we observed in the breakfast-exercise trial, may be due to differences in GLUT4 trafficking downstream of the signalling proteins we measured. Insulin-stimulated GLUT4 translocation and insulin sensitivity of skeletal muscle are increased *in vitro*, if muscle is pre-treated with insulin, without differences in Akt^{Ser473} or Akt^{Thr308} activation, or the transport activity of GLUT4 (18). This suggests priming of skeletal muscle by prior insulin exposure enhances subsequent insulin action. Pre-treatment with insulin *and* exercise augments this response, possibly because insulin (33) and exercise (28) stimulate GLUT4 translocation from different intracellular stores (8, 49). As such, in the current work, prior breakfast *and* exercise (the multiple stimuli in BE), may have enhanced skeletal muscle GLUT4 translocation during the OGTT. Although technically challenging future work should now quantify GLUT4 trafficking with fed *versus* fasted state exercise to confirm this. It is also possible that hepatic glucose disposal accounts for some of the increase in glucose disposal following BE *versus* FE. Similarly to skeletal muscle, insulin-stimulated hepatic glucose uptake is enhanced by prior exposure to insulin in dogs (44, 45). Thus, assuming that this response persists in humans and after exercise,

priming of the liver by prior breakfast could also contribute to greater glucose disposal during meals consumed post-exercise.

The increases in post-exercise glucose disposal with prior breakfast consumption were, however, offset by alterations in plasma glucose appearance. As such, postprandial glucose *concentrations* did not differ between the two exercise trials. Postprandial plasma glucose appearance rates are determined by three main factors: 1) the appearance of glucose from the meal; 2) residual appearance of glucose from previous meals; and/or 3) liver glucose output (glycogenolysis or gluconeogenesis). We showed that alterations in the postprandial plasma glucose appearance rate (Ra_{TOTAL}) after exercise with prior breakfast was mostly driven by increased appearance of glucose from the post-exercise OGTT. These alterations in the Ra_{TOTAL} suggest that differences in gut function (i.e. increased intestinal damage or absorptive capacity) and/or splanchnic blood flow altered the Ra_{TOTAL} . Thus, breakfast prior to exercise may alter postprandial glycemia via factors related to intestinal absorption and splanchnic handling of glucose, rather than just glucose metabolism by skeletal muscle.

There are several potential mechanisms which may explain the differences in glucose appearance rates due to intestinal absorption and/or splanchnic handling of glucose. Plasma I-FABP concentrations are used as a marker of damage to intestinal epithelial cells (60). We noted lower plasma I-FABP concentrations in the breakfast-exercise *versus* fasted-exercise trial, despite

increased plasma glucose appearance rates of the orally-ingested glucose post-exercise. It is therefore unlikely that increased intestinal damage was responsible for the higher plasma glucose appearance rates we observed with feeding *versus* fasting before exercise. The better maintenance of splanchnic perfusion during exercise with prior feeding is a likely explanation for this response (15). If splanchnic perfusion was better maintained during exercise with prior feeding, this may have also *directly* facilitated higher OGTT-derived glucose appearance rates in that trial *versus* the fasted-exercise trial. It should be acknowledged however, that this intestinal damage response may be specific to cycling, and could differ with other exercise modalities (e.g. running), within the context of pre-exercise feeding. An alternative mechanism for higher appearance rate of orally-ingested glucose with feeding before exercise could also be that apical glucose transporters were primed by the prior breakfast ingestion (40). Although further underlying mechanism(s) remain unclear, and should therefore be investigated with future work, we showed that a major determinant of post-exercise glycemia (plasma glucose appearance rates) are altered by pre-exercise feeding and that this is unlikely to be explained by increases in intestinal damage.

Our results show that the metabolic and intramuscular signaling responses to exercise conducted in a fed state cannot be readily inferred from responses observed with exercise in a fasted state. As well as a continual investigation of the mechanisms responsible for differences in postprandial glucose metabolism with altered pre-exercise feeding, future work should study whether the results

we observed are apparent with different post-exercise meals (including the co-ingestion of fat and protein). Moreover, if the acute alterations in postprandial metabolism translate into longer-term differences in insulin sensitivity with repeated bouts of exercise in the fed *versus* fasted state, and in overweight and obese populations, should now be a focus for future work.

To conclude, eating breakfast (*versus* fasting) before exercise increases post-exercise plasma glucose disposal rates but this is offset by increases in appearance rates of (mainly) orally-ingested glucose, a result that does not appear to be explained by a greater intestinal damage response to the exercise. We showed that pre-exercise breakfast consumption lowers insulinemia at meals that are consumed post-exercise, providing new evidence that the second meal effect is maintained even when exercise is performed between eating occasions.

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794

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995 TABLES

Table 2. Peak plasma concentrations, time to peak concentrations, and the time-averaged area under the curve (AUC) for various metabolites and hormones measured during the total within-lab period (300 min) and during an oral glucose tolerance test observation (120-min) that was conducted after breakfast followed by rest (Breakfast-Rest), breakfast followed by exercise (Breakfast-Exercise), or extended overnight fasting followed by exercise (Fasted-Exercise).

Age (y)	Stature (cm)	Breakfast- Rest	Breakfast- Exercise	Fasted- Exercise	
	Body mass (kg)				
	Peak glucose conc. (mmol·L ⁻¹)	10.62 (9.98, 11.25)	9.65 (9.27, 10.03) ^a	9.23 (9.42, 9.45)	<i>F</i> = 4.895, <i>p</i> = 0.03
	Body mass index (kg·m ⁻²)				
	Time to peak glucose (min)	36 (24, 47)	49 (36, 62)	49 (36, 61)	<i>F</i> = 2.588, <i>p</i> = 0.12
	Fat mass (kg)			10.6 (3.7)	
	Glucose AUC _{TOTAL} (mmol·L ⁻¹)	6.41 (6.21, 6.60)	6.05 (5.90, 6.15) ^a	6.07 (5.93, 6.27)	<i>F</i> = 6.126, <i>p</i> = 0.01
	Fat Mass Index (kg·m ⁻²) ¹			3.26 (1.12)	
	Body fat (%) ¹			14 (4)	
	Fat free mass (kg) ¹			65.5 (6.4)	
	$\dot{V}O_{2peak}$ (L·min ⁻¹) *			4.00 (0.72)	
	$\dot{V}O_{2peak}$ (mL·kg·min ⁻¹) *			53 (10)	
	Peak Power Output (W)			317 (67)	
	HR _{MAX} (beats·min ⁻¹)			189 (10)	

Data are presented as means and (standard deviation). $\dot{V}O_{2peak}$ = peak oxygen uptake. *n* = 12. **n* = 11, due to technical difficulties with the breath-by-breath analysis during one participant's preliminary testing. ¹ = derived by dual-energy x-ray absorptiometry (DEXA).

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Peak insulin conc. (pmol·L⁻¹)	286 (231, 341)	209 (148, 269)	282 (222, 337)	F = 3.900, p = 0.07
Time to peak insulin (min)	38 (26, 49)	56 (47, 65) ^a	43 (33, 54)	F = 4.579, p = 0.03
Insulin AUC_{TOTAL} (pmol·L⁻¹)	88 (79, 97)	62 (54, 71)^{aa}	76 (67, 85)	F = 9.28, p < 0.01
NEFA AUC _{TOTAL} (mmol·L ⁻¹)	0.16 (0.12, 0.19)	0.28 (0.24, 0.33) ^{aa}	0.37 (0.31, 0.42) ^{bb}	F = 24.98, p < 0.01
NEFA AUC_{OGTT} (mmol·L⁻¹)	0.10 (0.06, 0.14)	0.24 (0.20, 0.29)^{aa}	0.26 (0.22, 0.31)^{bb}	F = 15.51, p < 0.01
Triglyceride AUC _{TOTAL} (mmol·L ⁻¹)	0.85 (0.79, 0.91)	0.81 (0.75, 0.86)	0.75 (0.70, 0.79)	F = 4.319, p = 0.03
Triglyceride AUC_{OGTT} (mmol·L⁻¹)	0.84 (0.77, 0.92)	0.79 (0.72, 0.86)	0.70 (0.64, 0.77)	F = 4.677, p = 0.02
Lactate AUC _{TOTAL} (mmol·L ⁻¹)	1.05 (0.96, 1.13)	1.19 (1.08, 1.30)	1.16 (1.07, 1.27)	F = 3.645, p = 0.06
Lactate AUC_{OGTT} (mmol·L⁻¹)	1.12 (1.04, 1.20)	0.97 (0.89, 1.04)^{aa}	1.03 (0.96, 1.11)	F = 6.479, p = 0.02
β-hydroxybuturate AUC _{TOTAL} (mmol·L ⁻¹)	0.03 (0.00, 0.06)	0.08 (0.06, 0.13) ^a	0.14 (0.09, 0.19) ^b	F = 5.936, p = 0.03
I-FABP AUC_{TOTAL} (pg·mL⁻¹)	279 (242, 317)	304 (267, 366)	415 (353, 476)^{b cc}	F = 10.87, p < 0.01

Data are means and (normalized 95% confidence intervals). conc. = concentration; AUC_{TOTAL} = the time-averaged area underneath the concentration-time curve for the total within-lab period (300-min); AUC_{OGTT} = the time-averaged area underneath the concentration-time curve for the oral glucose tolerance test (120-min); I-FABP = intestinal fatty acid binding protein. *n* = 12 healthy men ^a represents a difference between breakfast rest and breakfast exercise, ^b a difference between breakfast rest and fasted exercise and ^c a difference between breakfast exercise and fasted exercise with *p* < 0.05. ^{aa}, ^{bb} or ^{cc} is the same difference between trials but with *p* < 0.01.

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LEGENDS TO FIGURES

Figure 1. Protocol schematic. An oral glucose tolerance test was conducted after breakfast followed by rest (BR), breakfast followed by exercise (BE), or extended overnight fasting followed by exercise (FE). Dual stable isotope tracers ([U-¹³C] glucose ingestion and [6,6-²H₂] glucose infusion) and muscle biopsies were combined to assess postprandial plasma glucose kinetics and intramuscular signaling, respectively.

Figure 2. The plasma glucose disposal rate (R_d) (**A**), metabolic clearance rate (**B**), the total rate of plasma glucose appearance (R_{aTOTAL}) (**C**), the rate of appearance of glucose in plasma from the oral glucose tolerance test (R_{aOGTT}) (**D**), and the plasma enrichments of [²H₂]-glucose (**E**) and [¹³C]-glucose (**F**) before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals. *n* = 12 healthy men. a = difference between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus* fasted exercise and c = breakfast exercise *versus* fasted exercise with *p* < 0.05.

Figure 3. Plasma glucose (**A**), plasma insulin (**B**), plasma non-esterified fatty acids (NEFA; **C**), plasma β-hydroxybutyrate (**D**) plasma triglyceride (**E**), and plasma intestinal fatty acid binding protein (IFAB-P; **F**) concentrations before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals. *n* = 12 healthy men. a = difference between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus* fasted exercise and c = breakfast exercise *versus* fasted exercise with *p* < 0.05.

Figure 4. The time-averaged (120-min) plasma glucose (**A**) and plasma insulin (**B**) incremental area under the curves (iAUC) and the Matsuda insulin sensitivity index (**C**; ISI_{MATSUDA}) for an oral glucose tolerance test conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals, with individual data shown as grey lines. *n* = 12 healthy men.

Figure 5. The phosphorylation of 5' AMP-activated protein kinase (**A**; phospho AMPK^{Thr172}, ratio p-AMPK to total-AMPK) and the phosphorylation of acetyl-CoA carboxylase (**B**; phospho ACC^{Ser79}, ratio p-ACC to total-ACC) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was

conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means \pm normalized 95% confidence intervals. $n = 9$ healthy men.

Figure 6. The phosphorylation of Akt2 (**A**; phospho Akt^{Ser473}, ratio p-Akt^{Ser473} to total-Akt2 and **B**; phospho Akt^{Thr308}, ratio p-Akt^{Thr308} to total-Akt2) and the phosphorylation of AS160 (**C**; AS160^{Thr642}, ratio p-AS160^{Thr642} to total-AS160) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means \pm normalized 95% confidence intervals. $n = 9$ healthy men.

Figure 7. Within-lab (300-min) substrate utilization after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Within-lab substrate utilization was calculated assuming that the plasma glucose disappearance rate was equivalent to plasma glucose utilization. Glycogen utilization during exercise (on breakfast exercise and fasted exercise only) was calculated as total carbohydrate utilization (from indirect calorimetry) minus plasma glucose utilization during exercise. This estimate of muscle glycogen utilization will therefore include the utilization of other non-glucose (e.g. lactate) carbohydrates. GLU = glucose; M-GLY = muscle glycogen; PRO = protein; CHO = carbohydrate. Data are presented as means \pm 95% confidence intervals. $n = 12$ healthy men.